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13. ABSTRACT (Maximum 200 words) Human exposure to chemicals is rarely limited to genotoxic agents but also to nongenotoxic chemicals. Nongenotoxic agents alter a cell's phenotype through epigenetic mechanisms at the transcriptional, posttranscriptional, translational, or posttranslational level. The integration of intercellular communication through gap junctions and intracellular pathways plays a role in maintaining the homeostasis by controlling the expression of genes that control cell proliferation, apoptosis, and differentiation. Our results showed that a series of USAFOSR-toxicants, such as polycyclic aromatic hydrocarbons and perfluorinated fatty acids were toxic by inhibiting gap junctional intercellular communication (GJIC). Specific structure-activity relationships of various PAH and PFFA isomers were the first structure-activity results reported for nongenotoxicants. The tumor promoting oxidants dicumyl peroxide, benzoylperoxide and H ₂ O ₂ reversibly inhibited GJIC at noncytotoxic doses but only in the presence of the antioxidant glutathione, whereas, the non-tumor promoting oxidant t-butylperoxide had no effect on these cell-signaling pathways. Ceramides reversibly inhibited GJIC only if they had an acyl group of 6-8 carbons. The C2-ceramide was a strong inducer of apoptosis while the GJIC inhibitory ceramides also inhibited apoptosis. In conclusion, epigenetic toxicants can alter intracellular signaling pathways that control mitogenesis and apoptosis and GJIC plays a role in modulating these pathways.			
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TERMINAL REPORT
(Nov. 1, 1996 - Feb. 28, 1999)

**“OXIDATIVE STRESS, SIGNAL TRANSDUCTION,
CELL-CELL COMMUNICATION”**

F49620-97-1-0022

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OBJECTIVES:

The basic hypothesis to be tested is that chemicals which produce oxidative damage in cells can trigger various signal transducing mechanisms, which, in turn, can inhibit gap junctional intercellular communication (GJIC) at the transcriptional, translational or posttranslational levels and inhibit apoptosis (programmed cell death).

Four specific aims were proposed to test this hypothesis:*

- Aim 1: To determine if a series of USAFOSR-toxicants can inhibit GJIC, using a series of human and rodent (for species comparison studies) cells in vitro, using the scrape-loading/dye transfer technique.
- Aim 2: To examine the potential mechanism by which an inhibitor of GJIC may have altered the redox state and altered the function of gap junctions.
- Aim 3: To examine if any of the chemicals which inhibit GJIC via some oxidative damage also inhibits apoptosis in the same cells.
- Aim 4: If time permits, we will attempt to remediate the toxic chemical or to prevent the toxicity (inhibition of GJIC) by some chemopreventive strategy.

*Due to reduction of budget, Aim 4 was eliminated and very selective experiments were done to examine Aims 1-3.

STATUS OF EFFORT:

This terminal report comes at the end of the second year of a three-year grant which was abruptly terminated. Definitive structural activity relationships were determined by correlating specific structural motifs of compounds of interest to the USAF with an effect on gap junction intercellular communication (**GJIC**) and mitogen activated phosphokinase (**MAPK**) pathways. Compounds tested were polycyclic aromatic hydrocarbons (PAHs), perfluorinated fatty acids (PFFAs) of which some are peroxisome proliferators that are well known non-genotoxic carcinogens, and chlorinated benzenes. The down regulation of GJIC and the activation of MAPK have been implicated in the induction of mitogenesis and tumor promotion. The results are as follows: (1a) Polycyclic aromatic hydrocarbons containing bay or bay-like regions were much more inhibitory of GJIC than the linear PAHs. (1b) The PAHs inhibitory of GJIC also activated the MAPK (we specifically looked at the extracellular receptor kinases ERK-1 and ERK-2). (1c) Inhibition of GJIC by these PAHs preceded the induction of MAPK. (1d)

These PAHs had no effect on the phosphorylation status of the gap junction proteins. (1e) The chlorinated isomers of anthracene, which are structurally similar but chemically different than the methyl isomers, inhibited GJIC at the same doses, times and time-recovery responses as the corresponding methyl-isomers of anthracene. (1f) Similarly these chlorinated anthracene isomers also activated MAPK with the same kinetics as the methyl isomers. (1g) Although MAPK was activated the phosphorylation status of the gap junction proteins was not altered. We used epidermal growth factor as the positive control for the inhibition of GJIC, induction of MAPK and the hyperphosphorylation of gap junction proteins, (1h) The GJIC inhibitory PAHs also induced arachidonic acid release from the membrane, whereas the non-GJIC inhibitory PAHs did not induce arachidonic acid release. The kinetics of arachidonic acid release was the same as those observed in the inhibition of GJIC (1i) The effect of a mixture of PAHs on GJIC was additive with no significant antagonistic or synergistic interactions. (2a) Perfluorinated fatty acids with a chain length of 7 to 10 carbons, which included perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA), were inhibitory to GJIC, but chain lengths of 2 to 5 carbons and 16 to 18 carbons were not very inhibitory to GJIC. (2b) PFDA, a GJIC inhibitor, activated MAPK whereas the non-GJIC inhibitor perfluoropentanoic acid did not activate MAPK. (2c) the kinetics of MAPK activation was slightly slower than the inhibition of GJIC. (3) Hexa-, tetra-, tri- and dichlorobenzene inhibited GJIC within 15 h and pentachlorobenzene inhibited GJIC within 5 min. The known tumor promoter and inducer of oxidative stress, pentachlorophenol, also reversibly inhibited GJIC and induced MAPK. (4) High doses of jet fuels (JP-4 and JP-8) also reversibly inhibited GJIC at noncytotoxic doses.

Many chemicals are also known to induce oxidative stress via the generation of H_2O_2 , such as the peroxisome proliferators PFOA and PFDA and polycyclic aromatic hydrocarbons. We, therefore, determined if oxidants such as H_2O_2 and organic peroxides also inhibit GJIC. H_2O_2 was shown to reversibly inhibit GJIC at a non-cytotoxic dose in glutathione (GSH) sufficient but not deficient cells. Hydrogen peroxide also hyperphosphorylated the gap junction proteins but this hyperphosphorylation did not correlate with GJIC activity. We have also transfected the WB rat liver epithelial cells with the H_2O_2 -generating enzyme uric acid oxidase. We began screening the clones for activity but due to the premature termination of the grant we had to end these efforts in order to complete other on going projects that were nearing completion. This system could provide an efficient way to quantitatively assess the endogenous effects of H_2O_2 on cell signalling mechanisms. The tumor promoting organic peroxides, benzoylperoxide and dicumylperoxide, both reversibly inhibited GJIC at non-cytotoxic doses, whereas the non-tumor promoting *t*-butylperoxide had no effect on GJIC. The inhibitory peroxides also activated MAPK and this activation followed inhibition of GJIC, but the

noninhibitory GJIC compound *t*-butylperoxide did not activate MAPK. Ceramides, which are endogenous signal transductants produced during oxidative stress, had differential effects on GJIC and apoptosis depending on the chain length of the acyl group. The C2 ceramide induced apoptosis but did not inhibit GJIC, whereas the C6 and C8 ceramide inhibited GJIC and apoptosis. The well-known tumor promoter TPA also inhibited C2 ceramide-induced apoptosis. Considering that these are the first direct evidence for the role of GJIC in apoptosis, we will repeat the effect of GJIC inhibitory ceramides on apoptosis one more time before publication. We will also determine the effect of the C2 ceramide, an inducer of apoptosis, on our non-communicating rat liver epithelial cell line.

ACCOMPLISHMENTS/NEW FINDINGS:

The down regulation of GJIC by environmental toxicants can result in chronic effects such as cancer, neurotoxicity and teratogenicity, depending on the cell type, tissue and organ that is exposed, as well as duration of exposure and time during development. Results on the structure-activity relationships contribute important information to a more mechanistic based risk assessment of non-genotoxic chemicals and directly fulfill the *goals of aim 1*. In particular, we have demonstrated that PAHs with bay or bay-like regions can inhibit GJIC that is followed by the activation of MAPK. **Although considerable research has been devoted to predicting the carcinogenicity of PAHs based on genotoxic properties and structurally alerting motifs, we were the first to identify structural motifs of PAHs that contribute to their epigenetic-toxic properties.** We are also the first to definitively show that the activation of MAPK does not result in the hyperphosphorylation of gap junctions. However, all of our GJIC inhibitors, including oxidative and non-oxidative compounds, also induce MAPK thereby suggesting that **MAPK and GJIC activity are inversely related and may share a common upstream intracellular pathway.** The data obtained from our *in vitro* results of chlorinated benzenes will be compared to the *in vivo* results of these same compounds done in Raymond Young's lab as an inter-USAFOSR collaboration. We also would like to note that the benzoylperoxide and dicumylperoxide but not *t*-butylperoxide were recently reported to be tumor promoters in SENCAR mice (Gimenez-Conti et al., Toxicol. Appl. Pharmacol. 149:73, 1998) which helps validate the use of our *in vitro* systems. We are also collaborating with Drs. Inoue and Sai from the Japanese Institute of Health in determining the underlying mechanism for the tumor promoting activity they observed with pentachlorophenol (PCP) and perfluoropentanoic acid. We have published a manuscript with our Japanese collaborators showing that PCP is a reversible inhibitor of GJIC. I will be going to Japan Feb. 13-28, 1999, to do *in vivo* experiments with PFOA and perfluoropentanoic acid correlating GJIC and MAPK activity with hepatomegaly and tumor promotion.

Although PFOA and PFDA are known to induce oxidative stress through the over production of H_2O_2 via peroxisome proliferation (Nemali et al., Toxicol. Appl. Pharmacol. **97**:72,1989) and, similarly, PAHs can generate H_2O_2 via oxidation by dihydrodiol dehydrogenase (Penning et al., Chem. Res. Toxicol. **9**:84, 1996, Flowers et al., Biochemistry **35**:13664, 1996), these compounds inhibited GJIC within 5 min which suggests that inhibition of GJIC can also be independent of oxidative stress. This short time period is not sufficient to induce peroxisome proliferation or significant oxidation of PAHs. Also the time period for the down regulation of GJIC by H_2O_2 is 1 h. **These are important observations since peroxisome proliferation is not always essential for tumor promotion by peroxisome proliferators.** Other peroxisome proliferators, such as nafenopen, WY-14,643, trichloroethylene and monoethylhexyl phthalate, are also known to directly down regulate GJIC (Fund. Appl. Toxicol. Suppl. (FATS) **36**:1134; FATS **30**:1054 &1068, Elcock et al, abstract#37, Intl. Meet. on Gap Junctions, Key Largo, 1997). In addition to the direct effects of these compounds on GJIC, H_2O_2 , a metabolic product of these compounds, can also reversibly down regulate GJIC at non-cytotoxic doses. The inhibition of GJIC by H_2O_2 requires GSH. **This is a very significant discovery demonstrating that the antioxidant GSH, is actually needed for the signal transduction mechanism of H_2O_2 -linked down-regulation of GJIC.** GSH was also shown to be involved in other signal transduction pathways such as the activation of NF-KB (Ginn-Pease, Biochem. Biophys. Res. Com. **226**:695, 1996) and the induction of c-jun (Kuo et al, Carcinogenesis **17**:815, 1996). GSH could be critical in supplying reducing equivalents to or conjugating with a key signal transductant or doing both. These results are directly related to the *goals of Aim 2*.

Ceramides are released from membranes during oxidative stress and are known to induce apoptosis. Therefore, we looked at the effects of various ceramides on GJIC and apoptosis. **The C2-ceramide induced apoptosis and had little effect on GJIC but the C6 and C8 ceramides as well as TPA inhibited GJIC and apoptosis. This is also a significant finding which implicates that GJIC is probably important in the transfer of a death signal and that different ceramides play contrasting signaling roles in the cells.** We also have preliminary results showing that GSH is partially involved in the down regulation of GJIC by the C6 and C8 ceramides. These inhibitory ceramides could be regulating GJIC, in part, through the same signaling pathway of H_2O_2 . H_2O_2 is also known to induce apoptosis and deplete the cells of GSH. Possibly the decrease in GSH levels under severe oxidative stress will prevent H_2O_2 from down regulating GJIC in the cell, thereby allowing the cell to undergo apoptosis. These results are directly related to the *goals of Aim 3*.

In summary, our results show distinct relationships between the structures of chemicals and their effects on GJIC and MAPK activity, which can play an important role in the predictive assessment of the epigenetic toxicity of chemicals. Further, we have advanced our knowledge that GSH, a key intracellular antioxidant, also plays a role in signal transduction relative to GJIC and have demonstrated that GJIC is also important in apoptotic events. In addition, the similarities in the inhibition of GJIC and the activation of MAPK among chemically different compounds suggest common biochemical pathways were involved.

A hypothetical link between these diverse chemicals may involve the peroxisome proliferator activated receptor α (PPAR α). Oxidants are well known for their ability to activate phospholipases and sphingomyelinase with consequent release of ceramides and fatty acids. With regards to PAHs, we have results indicating that the PAHs with bay-like regions induces the release of arachidonic acid. These metabolites, as well as the perfluorinated fatty acids are all known to form ligands with PPAR α . Therefore, the activation of PPAR- α may play an important role in the regulation of GJIC and MAPK independent of peroxisome proliferation. It would have been nice to test this hypothesis by determining the effect of PPAR antagonist and phospholipase inhibitor on the biological activity of our different compounds relative to GJIC and MAPK.

These recent findings have (a) demonstrated, again, that non-genotoxic chemicals can inhibit gap junctional intercellular communication (GJIC) at non-cytotoxic doses, in a dose dependent and reversal fashion with "threshold" characteristics; (b) these toxic chemicals can trigger signal transduction mechanisms which down regulate GJIC or induce apoptosis via non-oxidative and oxidative triggering mechanisms; and (c) by inhibiting GJIC, they can inhibit apoptosis or programmed cell death, stimulate the proliferation of initiated cells to act as tumor promoters, and block differentiation of normal cells to cause birth defects or block normal functions of differentiated cells to cause neurotoxicities.

These findings also are on the cutting edge of the field of "epigenetic toxicology" in that they are not only demonstrating the value of using GJIC as a "biomarker" for the practical use of screening and identifying potentially toxic chemicals that the US Air Force needs to know about, but also these findings are of use to the basic understanding of the biochemical mechanisms by which these chemicals work. This knowledge will give insights to preventive measures to take when exposed to such chemicals and to the modeling of realistic biological risk assessment models. It should be obvious that this approach to the characterization of the mechanisms of toxicity of non-mutagenic chemicals is directly relevant to the AF mission.

PERSONNEL SUPPORTED:

James E. Trosko, Ph.D. (P.I.); (15% - 12 months)
Brad L. Upham (Co-P.I) (75% - 12 months)
Hye-Kyung Na, Ph.D. (Res. Assoc.) (25% - 12 months)
Gang Chen, Ph.D. (Res. Assoc.) 5/97 (50% - 12 months)
Heather de Feijter-Rupp (Lab Manager) 14% - 12 months)
Nestor DeoCampo (Grad Student) (4% - 12 months)
Rebecca Cunningham (undergraduate student aid)

PUBLICATIONS:

1. Upham, B.L., L.M. Weis, A.M. Rummel, S.J. Masten, and J.E. Trosko (1996). The effects of anthracene and methylated anthracenes on gap junctional intercellular communication in rat liver epithelial cells. *Fund. Appl. Toxicol.* **34**, 260-264.
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16. Upham, B.L., T Koski, M.R. Wilson, and J.E. Trosko (1999). Differential effects of ceramides with various sizes of acyl groups on apoptosis and gap junctional intercellular communication (In Preparation).
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INTERACTIONS/TRANSITIONS:

A. Participation/Presentation at Meetings, Conferences, Seminars:

1. Upham, B.L., L.M. Weis, A.M. Rummel, S.J. Masten and J. Trosko (1996). The biological effects of methylated anthracenes on gap junctional intercellular communication depends on the ring position of the methyl group. (Presented at the 35th Annual Meeting of the Society of Toxicology), March 10, 1996, Anaheim, CA, *Fundam. Appl. Toxicol.* 30, 1066.
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- 27 Trosko, J.E. (1998). Modulated intercellular communication by chemicals and oncogenes: The mechanism of epigenetic toxicology. (Presented at L'Oréal Corp.), July 7, 1998, Paris, France.
- 28 Trosko, J.E. (1998). Modulation of gap junctional communication by oncogenes, tumor suppressor genes and epigenetic chemicals: The discovery of a "Biological Rosetta Stone". (Presented at Unite Mixte de Recherche, CNRS), July 9, 1998, Poitiers, France.
- 29 Trosko, J.E. (1998). Role of oncogenes, tumor suppressor genes and tumor promoters on the modulation of cell-cell communication: The discovery of a "Biological Rosetta Stone". (Presented at Karmanos Cancer Institute, Wayne State University), October 2, 1998, Ann Arbor, Michigan.
- 30 Trosko, J.E. (1998). Human pancreatic stem cells in development, cancer and diabetes. (Presented at Karmanos Cancer Institute, Wayne State University), October 2, 1998, Ann Arbor, Michigan.
- 31 Trosko, J.E. (1998). Discovery of a Biological Rosetta Stone in the understanding of the cause and treatment of cancer: The role of human stem cells and cell-cell communication. (Presented at Progetto Amazzone), Nov. 30-Dec. 4, 1998, Palermo, Italy.
- 32 Trosko, J.E. (1998). Discovery of a Biological Rosetta Stone in the understanding of the cause and treatment of cancer: The role of human stem cells and cell-cell communication. (Presented at the Medical Technology Program, Michigan State University), January 21, 1999, East Lansing, Michigan.
- 33 Upham, B.L., A.M. Rummel, M.R. Wilson, and J.E. Trosko (1999). Monomethyl or monochloro-isomers of anthracene with bay-like regions inhibited gap junctional intercellular communication and induced the mitogen activated phosphokinase ERK. (Presented at the 38th Annual Meeting of the Society of Toxicology), March 14, 1999, New Orleans, LA, Toxicological Sci. Suppl 00:000.

B. Consultant and Advisory Functions to other Laboratories:

1. Dr. Trosko consulted and collaborated with Dr. Tohru Inoue and Dr. Kimie Sai of the National Institute of Health Sciences in Tokyo. Our work involved *in vitro*/*in vivo* studies of the mechanisms of oxidative stress-inducing epigenetic toxicants.
2. Dr. Trosko initiated a collaboration with Dr. Emile Nuwaysir, NIEHS , using their new technology to measure altered gene expression with DNA chip technology.
3. Dr. Upham consulted for 3M Environmental Technology & Services, 3M Corp., St. Paul, MN, 6/1/96-6/1/97, Corporate contact was Robert Howell, (612) 778-7540. Consultation dealt primarily with the toxicological implication of exogenous modulation of gap junctional intercellular communication by perfluorinated aliphatic compounds.

C. Transitions:

The fundamental application of our findings is that the scientific field, in general, and the regulatory agencies (e.g., new EPA Cancer Guidelines) has acknowledged the role of GJIC in the tumor promotion phase of carcinogenesis. More and more chemical and drug companies (e.g., Sumitomo Chemical Corp., and Nippon Shinyaku Pharmaceutical Company of Japan) and government institutions (National Institute of Health Sciences of Japan and the U.S. EPA) have incorporated the concept of modulated GJIC as a marker for risk identification and assessment. Our fundamental concept and findings, namely that epigenetic toxic chemicals act by modulating gap junctional communication has finally been accepted by major chemical risk assessment modelers with the recent publication, (M. Rosenkranz, H. Rosenkranz and G. Klopman, "Intercellular communication, tumor promotion and non-genotoxic carcinogenesis: relationships based upon structural considerations", *Mutation Research* 381: 171-188, 1997).

NEW DISCOVERIES, INVENTIONS, PATENT DISCLOSURES:

None.

HONORS/AWARDS:

Dr. Trosko received the Kenneth P. DuBois Award from the Midwest Society of Toxicology Chapter (1995) and a Japan Science and Technology Award for a six-week consultantship to visit the National Institute of Radiological

Sciences in Chiba and the National Institute of Health Sciences in Tokyo
(Jan.-Feb., 1996)

Dr. Trosko was honored by the Society of Toxicology as the Plenary Speaker
in Nagoya, Japan, June 1998.

Dr. Upham received a Japan Food Hygiene Fellowship, to work for two weeks
(2/13/99-2/27/99) on a project titled "In vivo determination of changes in key
inter- and intra- cellular events involved in the mitogenic properties of
peroxisome proliferators" at the Biological Safety Research Center, Japanese
Institute of Health Sciences, Cellular & Molecular Toxicology Division,
Tokyo, Japan.